

## Na<sup>+</sup>/H<sup>+</sup> Exchange Inhibitor SM-20220 Improves Endothelial Dysfunction Induced by Ischemia-Reperfusion

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**ABSTRACT**—Endothelial cells play an important role in the physiologic homeostasis of the cerebral circulation. Previously, we showed that the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) inhibitor SM-20220 (*N*-(aminoiminomethyl)-1-methyl-1*H*-indole-2-carboxamide methanesulfonate) improved ischemic brain injury. In this study, we investigated the effect of SM-20220 on cerebrovascular dysfunction after ischemia-reperfusion, focusing on the kinds of dysfunction that involved endothelial function. In cultured bovine brain microvascular endothelial cells (BBMCs), the IC<sub>50</sub> value for the NHE activity of SM-20220 was  $4 \times 10^{-8}$  M. SM-20220 also reduced the cell injury induced by hypoxia/aglycemia-reoxygenation in BBMCs, with statistical significance at  $10^{-7}$  M ( $P < 0.05$ ). Next, the effect of SM-20220 on disruption of the blood-brain barrier and cerebral blood flow were evaluated using transient middle cerebral artery (MCA) occlusion models. Intravenous infusion of SM-20220 (0.4 mg/kg per hour for 1 h) attenuated the extravasation of Evans blue, a blood-brain barrier disruption indicator, into cerebral tissue on the day after transient ischemia ( $P < 0.05$ ). The occlusion of the MCA decreased the cerebral blood flow in the MCA territory by about 20%, and only about 45% of the preischemic value was recovered at 1-h reperfusion. A bolus injection of SM-20220 (1 mg/kg, i.v.) improved the postischemic hypoperfusion by about 75%, without causing changes in the systemic blood pressure. These results indicate that the protective effect of NHE inhibitor on ischemic brain injury may be at least partially mediated by the prevention of endothelial dysfunction.

**Keywords:** Na<sup>+</sup>/H<sup>+</sup> exchanger, Cerebral ischemia, Endothelial cell, Cerebral blood flow, Blood-brain barrier

The Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) is a mammalian plasma membrane glycoprotein that mediates the exchange of intracellular H<sup>+</sup> for extracellular Na<sup>+</sup> with a 1:1 stoichiometry and is thought to play an important role in the regulation of cell volume and pH<sub>i</sub> (1). The NHE has been found in all cell types tested, including neurons and glia. In the heart, this NHE inhibitor appears to elicit its cytoprotective effect against ischemic injury through the attenuation of Ca<sup>2+</sup> overload; that is, intracellular acidosis induced by ischemia/reperfusion may activate the NHE, causing an increase in intracellular Na<sup>+</sup>. Excessive accumulation of Na<sup>+</sup> leads to Ca<sup>2+</sup> overload via Na<sup>+</sup>/Ca<sup>2+</sup> exchangers (2). In brain tissue, it is thought that a similar process occurs in neurons and glia (3, 4), but it is yet not fully elucidated. Recently, we indicated that activation of the NHE plays a key role in the aggravation of ischemic injury in the brain as well as in the heart and that the NHE inhibitor SM-20220 (*N*-(amino-

iminomethyl)-1-methyl-1*H*-indole-2-carboxamide methane-sulfonate, Fig. 1) prevents ischemic brain damage such as that caused by infarction (5, 6) and reduces the increase in Na<sup>+</sup> content and the formation of edema (5). SM-20220 reduced the extent of hypoxia-reoxygenation injury in cultured neurons and glia (7). Furthermore, SM-20220 improves neurological outcome and survival rate (8) and may be a potential therapy for the treatment of acute stroke. Brain tissue consumes extremely high levels of oxygen and glucose, and maintenance of the cerebral blood flow is a prerequisite

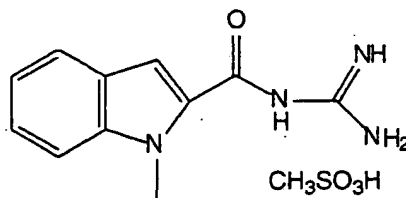


Fig. 1. Chemical structure of SM-20220.

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for the survival of neuronal tissue. It is well known that endothelial cells play an important role in the physiologic homeostasis of the cerebral circulation (9, 10). The NHE and  $\text{Na}^+/\text{Ca}^{2+}$  exchangers are present on cerebral vascular endothelial cells and maintain the ion homeostasis of these cells (11, 12). In this study, we investigated the effect of the NHE inhibitor SM-20220 on endothelial cell dysfunction induced by the ischemic condition.

## MATERIALS AND METHODS

### Materials

SM-20220 was obtained from Chemical Research Laboratories, Sumitomo Pharmaceuticals (Osaka). Polyoxyethylene sortitan monolaurate (Tween 20) and HEPES were purchased from Nacalai Tesque (Kyoto). Nigericin, urethane and poly-D-lysine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The Wako lactate dehydrogenase (LDH) test kits, Evans blue and acetoxymethylester of 2',7'-bis-2-carboxyethyl-5-(6)-carboxyfluorescein (BCECF) were obtained from Wako Pure Chemical Co. (Osaka). Basic fibroblast growth factor was obtained from Dainippon Pharmaceuticals (Osaka). Dulbecco's Modified Eagle's Medium (D-MEM), RPM1 medium 1640, penicillin/streptomycin and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Collagen type I was purchased from Nippon Becton Dickinson Co. (Tokyo). For the in vitro experiments, SM-20220 was dissolved in distilled water at  $10^{-3}$  M and diluted with culture medium. For the in vivo experiments, SM-20220 was dissolved in 8% (w/v) polyethylene glycol 400.

### Cultured cells

Normal bovine brain microvascular endothelial cells (BBMCs, Cat. No. CS-2BB3-C75) were purchased from Dainippon Pharmaceuticals. Cells were cultured in RPM1 Medium 1640 supplemented with 10% fetal bovine serum, 10 ng/ml basic fibroblast growth factor and 100 U/ml penicillin/streptomycin in collagen type 1-coated flasks, and they were maintained at 37°C in 5%  $\text{CO}_2$  in a humidified incubator. Cultures that had been passaged 3 to 5 times were used for the experiments.

### Measurement of NHE activity in endothelial cells

The activity of NHE inhibitor in BBMCs was determined by observing their ability to inhibit changes in the  $\text{pH}_i$  that were induced by an  $\text{NH}_4^+$  loading technique, as previously described (5). Briefly, the  $\text{pH}_i$  of BBMCs was measured using the fluorescent pH indicator dye BCECF (13). Cells were seeded in culture medium onto collagen type 1-coated cover glasses and loaded with 3 nM acetoxymethylester of BCECF (BCECF-AM) within 15 min of seeding. The fluorescence intensity of the dye was measured with excitation

pairs of 450 nm/490 nm and an emission wavelength of 530 nm, using a microscope-based microspectrofluorometer system (ARGUS-50; Hamamatsu Photonics, Hamamatsu). The fluorescence ratio 490 nm/450 nm was then calculated. Calibration of the  $\text{pH}_i$  was accomplished using the high  $\text{K}^+$ -nigericin method (14). The fluorescence ratio was plotted against  $\text{pH}_i$  and fitted by linear regression ( $r^2 = 0.988$ ).

After cells were loaded with BCECF-AM and a constant fluorescence ratio was observed, the cells were acidified by a transient (5 min) exposure to a HEPES-buffered, bicarbonate-free solution (composition: 10.0 mM HEPES, 137.0 mM NaCl, 5.9 mM KCl, 2.2 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$  and 14.0 mM glucose, pH 7.4) containing 20 mM  $\text{NH}_4\text{Cl}$ , followed by exchange into the same solution lacking  $\text{NH}_4\text{Cl}$ . The subsequent  $\text{pH}_i$  recovery from the acid load was measured. This measurement was performed under  $\text{HCO}_3^-$ -free conditions, in which the  $\text{pH}_i$  recovery from acidosis is restricted to NHE (15, 16). The inhibitory effect of SM-20220 on NHE activity was estimated by comparing the rate of  $\text{pH}_i$ -recovery in the presence and absence of the drug at 3 min after maximum acidification.

### Hypoxia/aglycemia-reoxygenation injury in endothelial cells

Hypoxia/aglycemia-reoxygenation was performed essentially as described by Lynch et al. (17). Briefly, cells were seeded onto collagen type 1-coated plates and cultured in D-MEM. Oxygen and glucose were then depleted by placing the cells in a hypoxic incubator (temperature: 37°C; atmosphere: 94%  $\text{N}_2$ , 5%  $\text{CO}_2$  and 1%  $\text{O}_2$ ). After 2 h, the medium was replaced with oxygenated D-MEM containing 5 mM glucose. Using an LDH test kit, the hypoxia/aglycemia-reoxygenation-induced cell damage was assessed throughout the hypoxia/aglycemia and the following reoxygenation periods by measuring the amount of LDH released into the culture medium (18), and this was expressed as a percentage of the total LDH. Total LDH was determined by measuring the amount of LDH released when all cells were destroyed by adding Tween 20 to the medium (0.1% (w/v) final concentration). SM-20220 was applied throughout the entire hypoxia/aglycemia and reoxygenation period. The normoxic control group was exposed to 2 h of normoxia/aglycemia followed by 24 h of normoxia/glycemia.

### Middle cerebral artery (MCA) occlusion in rats

All procedures related to the use of animals in the present study were reviewed and approved by the Institutional Animal Care and Use Committee at Sumitomo Pharmaceuticals Research Division and were in compliance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Male Wistar rats (Japan SLC, Hamamatsu), weight range about 200–240 g, were anesthetized with a gas mixture of

70% N<sub>2</sub>O, 30% O<sub>2</sub> and 1% halothane, and the surgical procedures were as described previously (5). Briefly, after median incision of the neck skin, the left external carotid artery and main carotid artery were occluded. Transient focal ischemia was induced by occluding the left MCA using an intraluminal suture technique (19). A 16-mm length of 4-0 nylon thread (outer diameter 0.45 mm), precoated with silicon (KE45W; Shinetsu, Tokyo), was inserted from the lumen of the internal carotid artery to occlude the left MCA at its origin. While the animals were anesthetized, the body temperature was maintained at 37°C with a heating pad (BWT-100; Bioresearch Center, Nagoya).

#### *Evaluation of the disruption of the blood-brain barrier (BBB)*

After the onset of MCA occlusion, anesthesia was discontinued and the existence of paresis of the right forelimb was used to assess ischemic insult. One hour after the onset of MCA occlusion, vehicle or SM-20220 was given as an i.v. infusion (5 ml/kg per hour) for 1 h using an infusion pump (model 100; KD Scientific, New Hope, PA, USA). Two hours after the onset of MCA occlusion, reperfusion was established by pulling the occluding thread, under anesthesia.

To evaluate the disruption of the BBB, the extravasation of Evans blue (EB), a BBB disruption indicator, into damaged tissue was measured by a modification of the method of Kawagoe et al. (20). Briefly, 21 h after the onset of MCA occlusion, rats were anesthetized with halothane and given an i.v. bolus injection (4 ml/kg) of saline containing 2% EB. Twenty-four hours after the onset of MCA occlusion, rats were anesthetized again with halothane and perfused transcardially with 100 ml of physiological saline (25°C at a pressure of 100 mmHg), prior to brain removal, to flush all blood components from the vasculature. The left MCA territory of the brain (1-mm anterior to 3-mm posterior to the bregma, (5)) was used for the determination of dye content. After their wet weight was measured, the brain pieces were soaked overnight in a tube containing 0.25 ml of 1 N KOH at room temperature. The alkaline solution was neutralized by adding 0.875 ml of 0.6 N H<sub>3</sub>PO<sub>4</sub>, and 1.625 ml of acetone was then added to the solution. The tube was shaken vigorously for a few seconds and spun centrifuged 3 times at 3000 rpm for 15 min. Absorbance of the extracted solution was measured by a spectrophotometer (U-2000; Hitachi, Tokyo) at 620 nm. The quantitative calculation of the dye content in the brain was based on a comparison with external standards (0.25–4 µg) that were diluted into an aliquot of the neutralized brain soaking solution.

#### *Measurement of cerebral blood flow (CBF)*

Under the anesthesia with a gas mixture of 70% N<sub>2</sub>O, 30% O<sub>2</sub> and 1% halothane, the right femoral artery was cannulated with a polyethylene catheter (PE50; Becton

Dickinson, Franklin Lakes, NJ, USA) for measuring the systemic blood pressure and heart rate and for removing blood samples for the analysis of blood gases and pH. Thirty minutes after the onset of MCA occlusion, vehicle or SM-20220 (1 mg/kg) was administered as an i.v. bolus injection (1 ml/kg). One hour after the onset of MCA occlusion, reperfusion was established by pulling the occluding thread. The blood flow of the cerebral cortex in the left MCA territory (5-mm lateral and 2-mm posterior to the bregma) was measured by a Laser-Doppler Flowmetry (PF-4001-2; Perimed, Järfälla, Sweden) with a PF-416 probe (Perimed). The CBF values at each time point (0, 5, 15, 30, 45, 60, 65, 75, 90, 105 and 120 min after the onset of MCA occlusion) were quantified with a Mac Lab 8 (Mk III; AD Instruments, NSW, Australia) and expressed as a percentage of the pre-ischemic value.

Systemic arterial blood pressure and heart rates were measured with a pressure transducer (P10EZ; Nihon Kohden, Osaka) through a blood pressure amplifier (AP-641G, Nihon Kohden), and blood gases and pH were measured with an oxygen analyzer (model 248; Ciba Corning, Tokyo).

#### *Statistical analyses*

All data are expressed as the mean ± S.E.M. of *n* observations. Statistical analyses were performed with the Statistical Analysis System (release 6.12; SAS institute Inc., Cary, NC, USA). Statistical comparisons between two groups were made by Wilcoxon's test or an F test followed by Welch's test or Student's *t*-test. Statistical comparisons between the vehicle and SM-20220 groups were made by Williams' multiple comparison (21). Differences were considered significant if *P* < 0.05.

## RESULTS

#### *Inhibitory effect on NHE activity in BBMCs*

The mean pH<sub>i</sub> of BBMCs in this series of experiments was 7.02 ± 0.02 (*n* = 118). Figure 2 shows the time course of the pH<sub>i</sub> in BBMCs. Addition of 20 mM NH<sub>4</sub>Cl caused pH<sub>i</sub> to rapidly increase due to the influx of NH<sub>3</sub> and the subsequent combination of most of these molecules with intracellular H<sup>+</sup>. After this rapid alkalinization, there was a acidification, caused by the entry of NH<sub>4</sub><sup>+</sup>, a small fraction of which dissociated to form NH<sub>3</sub> and H<sup>+</sup>. When the external NH<sub>4</sub><sup>+</sup> was removed, pH<sub>i</sub> rapidly fell as nearly all internal NH<sub>4</sub><sup>+</sup> dissociated to form NH<sub>3</sub> and H<sup>+</sup>. This measurement was performed under HCO<sub>3</sub><sup>-</sup>-free conditions, in which the pH<sub>i</sub> recovery from acidosis is restricted to NHE (15, 16). Removal of NH<sub>4</sub>Cl resulted in a rapid intracellular acidification of about 0.8 pH units. The pH<sub>i</sub> rapidly recovered in the absence of drugs, reaching its normal value at 5 min after acidification (Fig. 2: a and c). SM-20220 inhibited the recovery of the pH<sub>i</sub> in BBMCs with IC<sub>50</sub> values for

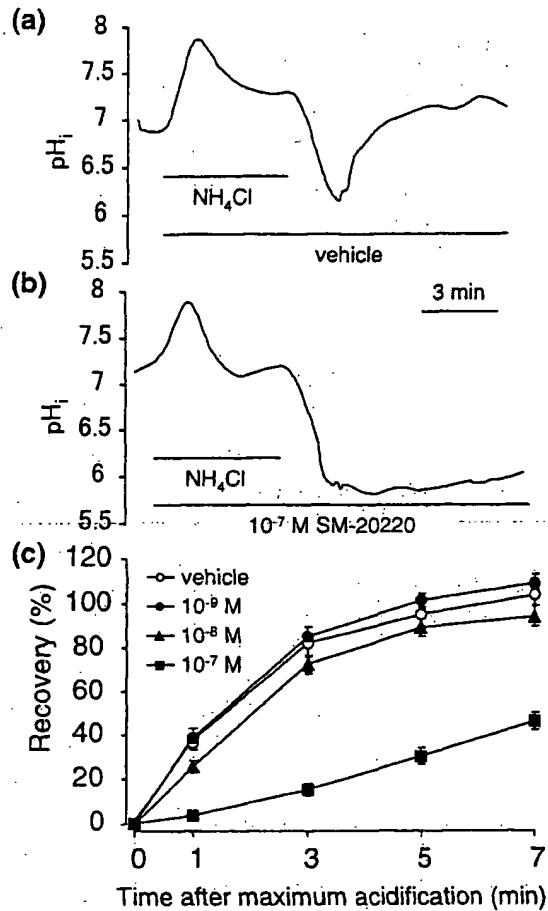


Fig. 2. The pH<sub>i</sub> recovery from NH<sub>4</sub><sup>+</sup>-induced acidification in bovine brain microvascular endothelial cells (BBMCs). The cells were acidified by a transient (5 min) exposure to 20 mM NH<sub>4</sub>Cl followed by removal of the NH<sub>4</sub>Cl and the pH<sub>i</sub> was measured using the fluorescent pH indicator acetoxymethyl ester of 2',7'-bis-2-carboxyethyl-5-(6)-carboxyfluorescein (BCECF). Panels a and b are typical tracings of pH<sub>i</sub> in the absence and presence of SM-20220, 10<sup>-7</sup> M, respectively. pH<sub>i</sub> recoveries in the absence and presence of SM-20220 (10<sup>-9</sup>–10<sup>-7</sup> M) are summarized in panel c. Each point represents the mean ± S.E.M. of 28–34 cells.

inhibition of the recovery at 3 min after acidification of  $4 \times 10^{-8}$  M (Fig. 2: b and c).

#### Effect on hypoxic injury in BBMCs

In BBMCs, an approximately threefold increase in LDH release was observed after 2 h of hypoxia/aglycemia, compared with the non-hypoxic group (non-hypoxia:  $8.1 \pm 2.8\%$  and hypoxia/aglycemia:  $26.6 \pm 3.6\%$ , Fig. 3a). Twenty-four hours of reoxygenation enhanced this increase in LDH release in the experimental group but not in the non-hypoxic group (non-hypoxia:  $8.4 \pm 3.2\%$  and hypoxia/aglycemia-reoxygenation:  $68.3 \pm 5.1\%$ , Fig. 3b).

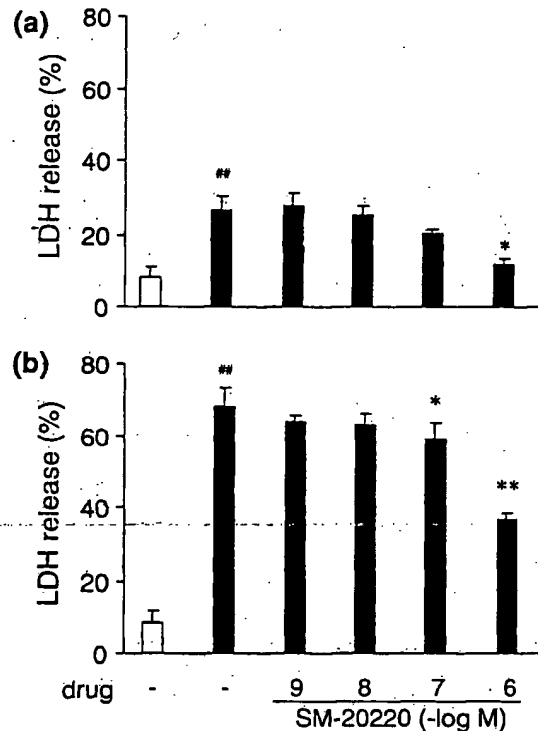


Fig. 3. Effect of SM-20220 on lactate dehydrogenase (LDH) release induced by hypoxia/aglycemia (a) or hypoxia/aglycemia-reoxygenation (b) in BBMCs. BBMCs were subjected to 2 h of hypoxia/aglycemia followed by 24 h of reoxygenation (closed column). The non-hypoxia group (open column) was exposed to 2 h of normoxia/aglycemia followed by 24 h of normoxia/glycemia. Each column represents the mean ± S.E.M. of 5 separate experiments. \*\* $P < 0.01$ , statistical significance of the difference from the non-hypoxia group by Student's *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ : statistical significance of the difference from the hypoxia/aglycemia or hypoxia/aglycemia-reoxygenation and vehicle treatment group by Williams' multiple comparison.

SM-20220 reduced the hypoxia/aglycemia-induced LDH release in a concentration-dependent manner, and this effect was statistically significant at 10<sup>-6</sup> M (Fig. 3a). SM-20220 also reduced the hypoxia/aglycemia-reoxygenation-induced LDH release in a concentration-dependent manner, and this effect was statistically significant in the range of 10<sup>-7</sup> M to 10<sup>-6</sup> M (Fig. 3b). At a concentration of 10<sup>-6</sup> M, SM-20220 reduced the hypoxia/aglycemia-reoxygenation-induced LDH release by 45%.

#### Effect on the disruption of BBB induced by ischemia/reperfusion

Twenty-four hours after the onset of MCA occlusion, the vehicle treatment group showed marked extravasation of EB, an indicator of BBB disruption, into the injured brain; there was, however, no visible dye extravasation into the

brain in the region contralateral to the hemisphere of MCA occlusion. The dye content of the sham-operated and MCA occlusion (vehicle) groups were  $2.2 \pm 0.3$  and  $7.4 \pm 0.7$   $\mu\text{g/g}$  wet tissue, respectively (Fig. 4). Intravenous infusion of SM-20220 (0.4 mg/kg) given after occlusion of the MCA significantly suppressed the extravasation of EB into the brain ( $4.9 \pm 0.4$   $\mu\text{g/g}$  wet tissue, Fig. 4).

#### Effect on CBF

MCA occlusion rapidly decreased the CBF by 20%, and only about 45% of the preischemic value was recovered when the occluder was removed (Fig. 5b). An intravenous bolus injection of SM-20220 (1 mg/kg, i.v.) after 30 min of the onset of MCA occlusion did not affect the CBF during ischemia, but markedly improved the postischemic hypoperfusion to more than 75% of the preischemic value, without affecting the systemic blood pressure or the arterial partial pressure of O<sub>2</sub> and CO<sub>2</sub> or pH (Fig. 5a, Table 1).

#### DISCUSSION

Our previous study indicated that activation of the NHE plays a important role in ischemic injury to the brain as well as the heart, and that the NHE inhibitor SM-20220 prevents ischemic brain damage (5). Specifically, in MCA occlusion models, SM-20220 given as an i.v. bolus injection in the range of 0.3 – 1.0 mg/kg attenuated the edema formation, increase in Na<sup>+</sup> content, and infarction size induced by 2 h of MCA occlusion followed by reperfusion. In cultured neurons and glia, SM-20220 prevented hypoxic injury (7),

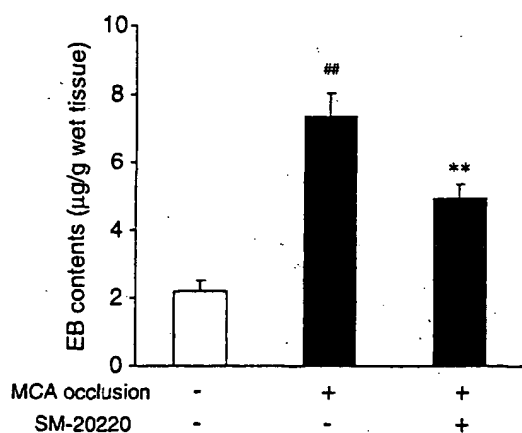


Fig. 4. Effect of SM-20220 on the extravasation of Evans blue (EB) in a transient middle cerebral artery (MCA) occlusion model. SM-20220 was administered as an i.v. infusion for 1 h (0.4 mg/kg per hour) at 1 h after the onset of occlusion. Each column represents the mean  $\pm$  S.E.M. of 15–16 animals. <sup>#</sup> $P < 0.01$ : statistical significance of the difference from the sham-operated group by Welch's test. <sup>\*\*</sup> $P < 0.01$ : statistical significance of the difference from the MCA occlusion and vehicle treatment group by Student's *t*-test.

and this result indicates that SM-20220 is a neuroprotective agent.

Endothelial cell dysfunction is one of the most important factors in ischemia-reperfusion-induced tissue injury. It is known that the endothelial dysfunction in the brain causes the decrease in the CBF after reperfusion, the disruption of the BBB and leukocyte adhesion to endothelial cells (9). It is also known that the protection of endothelial cells improves ischemic damage in stroke patients (22). There have been no reports concerned with the role of NHE in the endothelial dysfunction induced by brain ischemia and we investigated it using SM-20220.

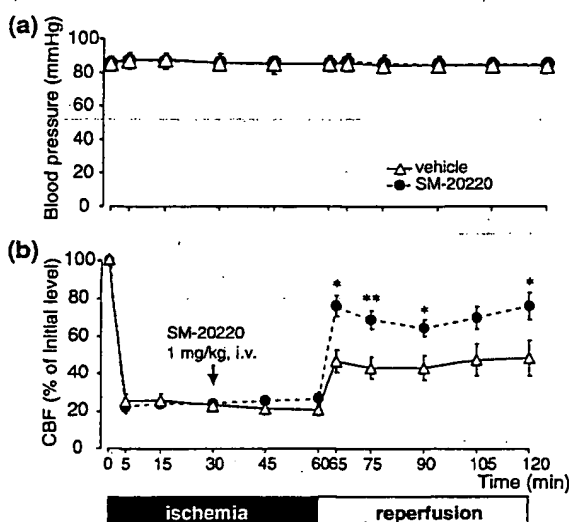


Fig. 5. Effect of SM-20220 on the systemic blood pressure (a) and the blood flow of the cerebral cortex (CBF, b) in a transient ischemia model. SM-20220 (1 mg/kg, i.v.) was bolus injected at 30 min after the onset of MCA occlusion. Each point represents the mean  $\pm$  S.E.M. of 7 animals. <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ : statistical significance of the difference from the vehicle group by Student's *t*-test.

Table 1. Arterial blood pH, partial pressure of CO<sub>2</sub> (PCO<sub>2</sub>) and O<sub>2</sub> (PO<sub>2</sub>) during the measurement of cerebral blood flow

		Before ischemia	One hour after reperfusion
Vehicle (n = 7)	pH	7.46 $\pm$ 0.01	7.45 $\pm$ 0.01
	PCO <sub>2</sub> (mmHg)	40 $\pm$ 1	41 $\pm$ 1
	PO <sub>2</sub> (mmHg)	92 $\pm$ 2	88 $\pm$ 2
SM-20220 (n = 7)	pH	7.45 $\pm$ 0.01	7.44 $\pm$ 0.01
	PCO <sub>2</sub> (mmHg)	41 $\pm$ 1	43 $\pm$ 2
	PO <sub>2</sub> (mmHg)	89 $\pm$ 2	95 $\pm$ 6

Rats were subjected to 1-h occlusion of the middle cerebral artery followed by 1 h of reperfusion. SM-20220 (1 mg/kg) was intravenously bolus injected at 30 min after the onset of occlusion. No statistical significance was observed between the vehicle and SM-20220 groups in each parameter by Student's *t*-test.

In the first experiment, we determined the potency of SM-20220's inhibition of the NHE activity and its cytoprotection against hypoxia-reoxygenation-induced injury in BBMCs. These cerebral microvascular endothelial cells have different physiologic properties from those of endothelial cells derived from other tissues (9, 10, 23). The  $pH_i$  in cerebral microvascular endothelial cells has been reported in only a few papers, and none of those used cells derived from a bovine source. Usually, the  $pH_i$  is regulated largely by the NHE and the  $Na^+$ -dependent  $HCO_3^-$ - $Cl^-$  anion exchanger (for a review, see Roos and Boron (24)). We observed the steady-state  $pH_i$  of BBMCs to be 7.02 when the cells were continuously superfused with medium that did not contain  $HCO_3^-/CO_2$ , which was used to block the  $Na^+$ -dependent  $HCO_3^-$ - $Cl^-$  anion exchanger. Hsu et al. (11) reported that the  $pH_i$  in piglet cerebral microvascular endothelial cells superfused with  $HCO_3^-/CO_2$ -containing medium was 7.18. The slightly lower  $pH_i$  we observed in BBMCs may have been due to the different kind of superfusing medium rather than the different species; in fact, blocking the  $Na^+$ -dependent  $HCO_3^-$ - $Cl^-$  anion exchanger via the application of inhibitor or  $Cl^-$ -free culture medium slightly lowers the steady-state  $pH_i$  (11). In the present study, the  $IC_{50}$  value of SM-20220 for the inhibition of NHE in BBMCs was  $4 \times 10^{-8}$  M. On the other hand, we showed that the  $IC_{50}$  values of SM-20220 for the inhibition of NHE in neurons and glia, which were determined by same method, were  $5 \times 10^{-9}$  and  $2 \times 10^{-8}$  M, respectively (5). Thus, the inhibitory activity of SM-20220 for NHE in endothelial cells was nearly equal or slightly weaker compared with those in neurons and glia.

In the non-hypoxic group, BBMCs released only a very small amount of LDH during the 24-h reoxygenation period compared with 2 h of hypoxia/aglycemia. It has been reported that brain microvascular endothelial cells derived from rats are resistant to short periods (<24 h) of hypoxia alone, but that they are damaged by prolonged hypoxia that is accompanied by hypoglycemia (25). Deprivation of glucose inhibits ATP production, which explains why LDH is released only under aglycemic conditions. The protective effect of SM-20220 on hypoxic injury in BBMCs could reflect its inhibitory action on the NHE and the minimum effective concentration of SM-20220 on hypoxia/aglycemia-reoxygenation injury was  $10^{-7}$  M. We strongly believed that endothelial cells would be one of the important targets for the cerebroprotective effect of the NHE inhibitor, because the minimum effective concentrations of SM-20220 to protect against hypoxia/aglycemia-reoxygenation injury to neurons and glia were  $10^{-8}$  and  $10^{-7}$  M, respectively (7).

To clarify this indication, we next investigated the effect of SM-20220 on ischemia-induced endothelial dysfunctions in vivo models.

EB is a dye that binds to serum albumin and often used to

estimate the BBB permeability (20). The BBB is formed by a complex cellular system of endothelial cells, astroglia, pericytes, perivascular macrophages, and a basal lamina; and the lumen of the cerebral capillaries is covered by endothelial cells that form narrow, intercellular tight junctions (11). SM-20220 at 0.4 mg/kg attenuated the EB extravasation into brain tissue induced by transient ischemia. This result indicates that SM-20220 protects endothelial dysfunction in vivo. In a similar model, SM-20220 reduced brain infarction and the effective minimum dosage was 0.4 mg/kg (26). Thus, the protective effect of SM-20220 against endothelial dysfunction could be involved in the mechanisms through which this compound reduces brain injury. This effect is also supported by our recent study that SM-20220 attenuates the increase in leukocyte accumulation in brain tissue induced by ischemia-reperfusion (26).

It is indicated that many factors related to endothelial cell injury contribute to the formation of "postischemic hypoperfusion" or the "no-reflow phenomenon". For example, vasoconstriction mechanisms mediated by endothelin, and attenuated by nitric oxide, have been suggested (for a review, see Akopov et al. (27)). In our model, the CBF after reperfusion decreased more than 50% compared with the preischemic value and this level was sustained until 1 h after reperfusion. Thus, this hypoperfusion may reflect ischemic injury rather than reperfusion injury. SM-20220 given at the same dosage that prevented edema formation and infarction in our previous study (5) attenuated this hypoperfusion by about 75%. These results suggest that SM-20220 may improve the postischemic hypoperfusion through the protection of endothelial dysfunction during ischemia.

In summary, we investigated the effect of SM-20220 on the endothelial dysfunction induced by ischemia. SM-20220 directly prevented hypoxic endothelial cell injury. SM-20220 also protected against disruption of the BBB and postischemic hypoperfusion. These results indicate that the protective effect of NHE inhibitor on ischemic brain injury may be at least partially mediated by the prevention of endothelial dysfunction.

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